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DRUG PROTEIN CONJUGATES—I. A STUDY OF THE COVALENT BINDING OF [^{14}C]CAPTOPRIL TO PLASMA PROTEINS IN THE RAT

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Abstract—The metabolism of [^{14}C]captopril has been investigated *in vitro* and *in vivo* in male Wistar rats. The formation of conjugates of [^{14}C]captopril with plasma proteins was observed both *in vitro* and *in vivo*: 180 min after intravenous infusion of [^{14}C]captopril 35 ± 5% of total radioactivity was covalently bound to plasma proteins. The fate of [^{14}C]captopril–plasma protein conjugates was investigated *in vivo*. [^{14}C]Captopril was incubated *in vitro* with rat and human plasma and the resulting captopril–protein conjugates were infused into male rats. The plasma concentration of [^{14}C]captopril–rat plasma protein conjugates declined monoexponentially with a half-life of 71.1 ± 2.2 min. After 180 min 28 ± 3% of the radioactivity was excreted in urine, largely as [^{14}C]captopril–cysteine mixed disulphide (67%). Thus although captopril readily forms covalent bonds with plasma proteins the resulting conjugates dissociate *in vivo*. The toxicological implications of these findings are discussed.

Captopril (D-3-mercapto-3-methyl-propanoyl-L-proline) is a novel angiotensin I converting enzyme inhibitor, which is used for the treatment of various forms of hypertension and congestive heart failure [1, 2]. However, a number of adverse reactions to this drug have been reported, including skin rash, fever, loss of taste, proteinuria, glomerulonephritis, serum sickness, agranulocytosis and ulcers [1, 3–8]. Moreover, immune complexes have been detected in the glomerular basement membrane in renal biopsy samples from patients taking captopril [9]. The nature and the time course of the adverse reactions are consistent with there being an underlying immunological mechanism, suggesting that captopril may either function as a hapten or that it pharmacologically modulates the immune system. *In vitro* studies have shown that captopril forms covalent bonds with plasma proteins [10]. We have, therefore, investigated the relationship between the metabolism of captopril and the formation of captopril plasma protein conjugates *in vivo*, in order to gain further understanding of the toxicity of captopril.

MATERIALS AND METHODS

The [^{14}C]captopril used in these studies was labelled in the amide carbonyl (5.02 μCi/mg) and was a gift from Dr. B. H. Migdalof of the Squibb Institute, New Brunswick, NJ. who also supplied authentic samples of captopril disulphide, captopril cysteine mixed disulphide, captopril glutathione mixed disulphide and *S*-methyl captopril. Silica gel thin layer chromatography plates (20 × 20 × 0.2 cm) were obtained from British Drug Houses, Poole, U.K. and scintillant (NE 260) and NCS tissue solubiliser from Nuclear Enterprises. The radioactive content of all samples was determined using an Intertechnique SL 30 liquid scintillation spectrometer; counting efficiency was determined with automatic external standardisation and the use of previously prepared quench curves. General reagents were obtained from British Drug Houses and Sigma Chemical Co. (London, U.K.). All solvents were redistilled before use. Male Wistar rat plasma was obtained from blood samples, withdrawn from the tail artery into heparinised containers, under ether anaesthesia. Human male plasma was obtained from blood samples taken from the antecubital vein into heparinised containers.

Determination of [^{14}C]*captopril metabolites and* [^{14}C]*captopril protein conjugates in plasma.* Plasma, from either *in vitro* or *in vivo* experiments, was mixed thoroughly with *N*-ethylmaleimide (2–4 mg/ml), to derivatise free captopril [11], and then extracted with 3 vol. methanol. The protein precipitate was extracted twice with methanol (3 vol.) and the extracts combined. An aliquot was taken to determine total methanol extractable radioactivity; the remainder was concentrated to 0.1 ml, in a stream of nitrogen, and then separated by thin layer chromatography) (TLC) together with authentic standards of captopril *N*-ethylmaleimide derivative, captopril disulphide, captopril cysteine mixed disulphide, captopril glutathione mixed disulphide and *S*-methyl-captopril. The relative proportions of captopril and its metabolites present were determined essentially as previously described [10, 11].

The amount of [^{14}C]captopril covalently bound to plasma proteins was determined by the method of Sun and Dent [12]. Briefly, the methanol precipitated proteins were dissolved in 0.1 M phosphate buffer (pH 7.4) containing 2% sodium dodecyl sulphate (SDS) by boiling the solution for 10 min, and then

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dialysed against one litre of 0.01 M phosphate buffer (pH 7.0) containing 0.1% SDS for 24 hr. The radioactive content of an aliquot of the dialysed sample and an aliquot of the dialysis buffer were then determined by liquid scintillation spectrometry. The amount of covalently bound radioactivity remaining in the dialysis bag was determined by subtracting the amount of radioactivity in the same volume of dialysis buffer [12]. Any procedural losses of plasma proteins were corrected by determining plasma proteins [13] before extraction and after dialysis (protein loss was always less than 10%).

In vitro *metabolism of* [^{14}C]*captopril.* [^{14}C]Captopril (0.20 mg; 0.02 μCi) was incubated with rat or human plasma (1 ml) in a shaking water bath at 37°. Immediately after mixing (control), and after 10, 30 and 60 min incubation, *N*-ethylmaleimide (2 mg/ml) was added, and after mixing [^{14}C]captopril metabolites and [^{14}C]captopril covalently bound to plasma proteins were determined as described above.

In vivo *metabolism of* [^{14}C]*captopril.* Male Wistar rats (250–300 g) were anaesthetised with urethane (14% in saline; 10 ml/kg i.p.) and the trachea, carotid artery and the jugular vein cannulated with polypropylene tubing of the appropriate size. After heparinisation of the animals (400 units/kg), [^{14}C]captopril (5 μCi, 4 mg/kg) in saline (1.6 ml/kg) was infused over 2 min via the jugular vein, and blood samples collected at 5, 30, 60 and 120 min; plasma was obtained immediately by centrifugation and *N*-ethylmaleimide (4 mg/ml) added.

Blood was replaced with an equal volume of physiological saline. [^{14}C]Captopril metabolites and [^{14}C]captopril covalently bound to plasma proteins were determined as described above.

At 3 hr the animals were killed, a terminal blood sample obtained and the liver, lungs, kidneys and spleen removed for the determination of radioactive content. Aliquots of tissue (50 mg) were dissolved in NCS tissue solubiliser (1 ml) at 50° overnight, neutralised with acetic acid, and then dissolved in scintillant (12 ml) and the tissue radioactivity determined.

Urine was aspirated from the bladder at 3 hr into *N*-ethylmaleimide (2 mg/ml). [^{14}C]Captopril metabolites were determined by liquid scintillation spectrometry, after separation by TLC as described above for plasma.

Preparation of [^{14}C]*captopril protein conjugates.* Rat or human plasma (1 ml) was incubated with [^{14}C]captopril (0.2 mg/1 μCi) at 37°, with shaking, overnight. The plasma was then diluted with 0.9% saline (4 ml) and concentrated to 1 ml in an Amicon B15 protein concentrator. This process was repeated 3 times. The Amicon B15 concentrator has a molecular weight cut-off at 15,000. The amount of [^{14}C]captopril covalently bound to plasma proteins after incubation was determined as described above.

Metabolism of [^{14}C]*captopril plasma protein conjugates* in vivo. Male Wistar rats (250–300 g) were anaesthetised and cannulated as described for the [^{14}C]captopril metabolism experiment. Rat or human plasma (1 ml) containing covalently bound [^{14}C]captopril (0.35 μCi/ml rat plasma proteins; 0.25 μCi/ml human plasma proteins) was infused i.v. into the jugular vein over 4 min. Blood samples (1 ml) were obtained from the carotid artery at 5, 30, 60, 120 and at 180 min, when the animals were killed, for the determination of [^{14}C]captopril metabolites and [^{14}C]captopril covalently bound to plasma proteins as described above. The radioactive content of the lungs, liver, kidney and spleen, at 3 hr, were also determined as described before. Urine was aspirated from the bladder at 3 hr for the determination of total radioactivity and of [^{14}C]captopril metabolites.

Calculations. The half-life of [^{14}C]captopril covalently bound to either rat or human plasma proteins was calculated by least squares regression analysis of the plasma concentration vs time curve. The apparent volume of distribution was calculated by dividing the dose administered by the extrapolated value of the covalently bound drug concentration at time zero, followed by correction for the infusion time by the method of Loo and Riegelman [21]. For statistical analysis a non-paired Student's *t*-test was used. All results are expressed as the mean ± S.E.M.

RESULTS

The covalent binding of [^{14}C]captopril to rat (Fig. 1(a)) and human (Fig. 1(b)) plasma proteins *in vitro* increased with time and after 60 min 60% and 28% was covalently bound to rat and human plasma proteins respectively. Extraction with methanol, followed by equilibrium dialysis in SDS [12] removed 98.5% of the radioactivity from control incubations to which *N*-ethylmaleimide was added directly after mixing of [^{14}C]captopril with plasma proteins. Also formed were captopril disulphide and polar metabolites of captopril which are thought to be mixed disulphides of captopril with glutathione and cysteine [10]. These results are similar to those reported by Wong *et al.* [10], who used ultrafiltration and acid precipitation techniques to determine radioactivity covalently bound to protein.

From Fig. 2 it can be seen that [^{14}C]captopril becomes extensively and covalently bound to plasma proteins *in vivo*; after 3 hr approximately 34% of the radioactivity in plasma was covalently bound to plasma proteins. Polar metabolites of captopril (captopril gluthathione mixed disulphide and captopril cysteine mixed disulphide) and *S*-methyl captopril were identified in plasma by co-chromatography with authentic standards [10, 11]. The plasma concentration–time profile of [^{14}C]captopril was similar to that reported for man [14], and appeared to be curvilinear; hence a plasma half-life was not obtained.

Drug protein conjugates of [^{14}C]captopril with rat plasma proteins and human serum proteins were synthesised *in vitro*. After purification, at least 94% of the [^{14}C]captopril was assessed to be covalently bound; the amount of captopril bound to rat plasma and human plasma was 0.35 μCi (0.07 mg/ml) and 0.25 μCi (0.05 mg/ml), respectively.

The plasma concentration–time curve of [^{14}C]captopril covalently bound to rat plasma proteins and to human plasma proteins is shown in Fig. 3; the plasma concentration–time curve of free [^{14}C]captopril is shown for comparison.

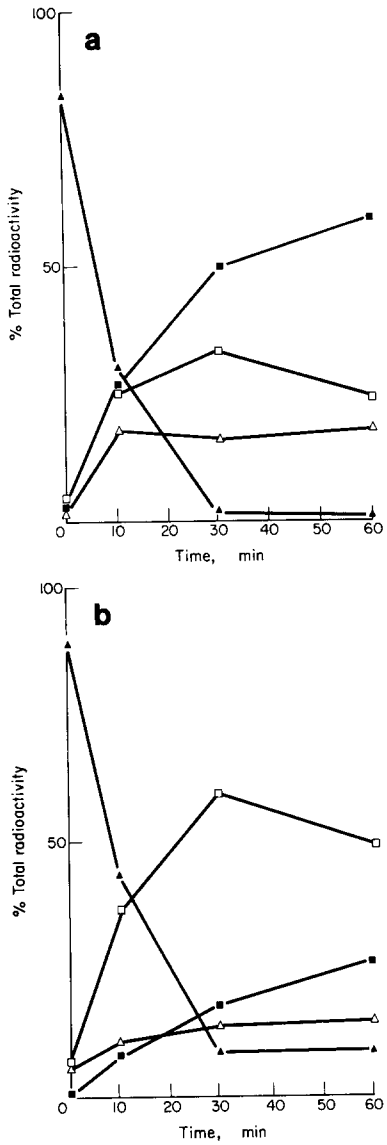

Fig. 1. The relative concentrations of [^{14}C]captopril (▲), [^{14}C]captopril disulphide (△), [^{14}C]captopril covalently bound to plasma proteins (■) and polar metabolites of [^{14}C]captopril (□), after incubation of [^{14}C]captopril with (a) rat serum and (b) human plasma *in vitro*. The results are means of 3 individual experiments, S.E. < 10%.

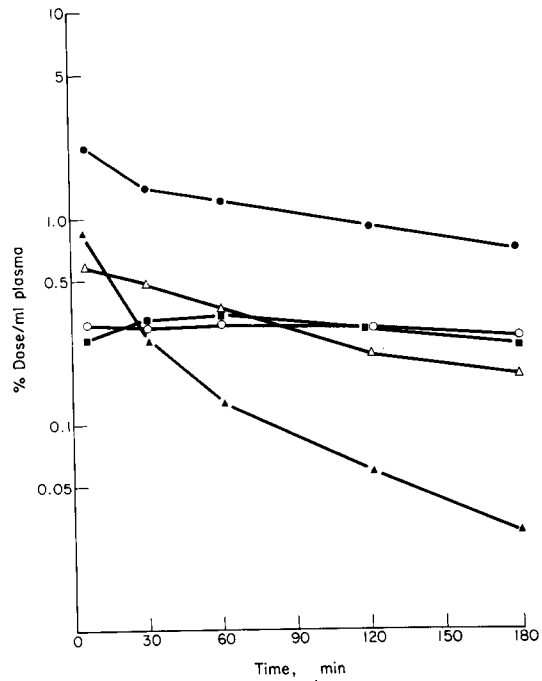

Fig. 2. Concentrations in plasma of [^{14}C]captopril (▲), polar metabolites of [^{14}C]captopril (△), *S*-methyl [^{14}C]captopril (○), [^{14}C]captopril covalently bound to plasma proteins (■), and total radioactivity (●), after administration of [^{14}C]captopril to rats. Results are means of 5 experiments, S.E. < 10%.

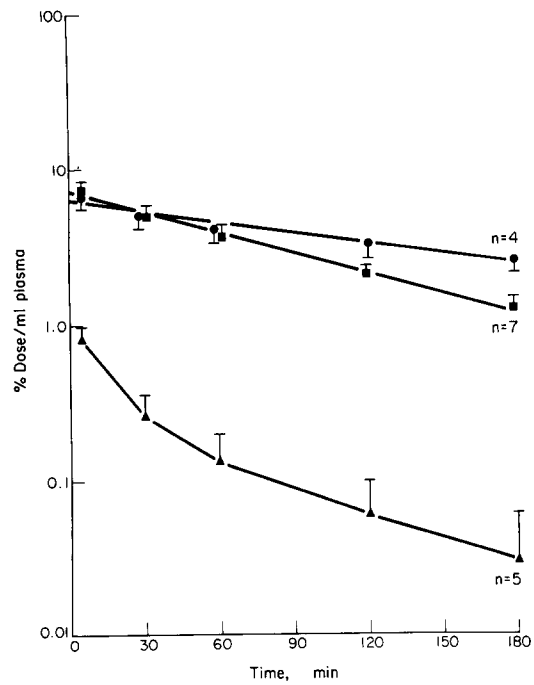

Fig. 3. Concentrations in plasma of [^{14}C]captopril covalently bound to plasma proteins after administration of [^{14}C]captopril covalently bound to rat plasma proteins (■) and after administration of [^{14}C]captopril covalently bound to human plasma proteins (●). The plasma concentration profile of [^{14}C]captopril, after administration of free [^{14}C]captopril (▲) is shown for comparison.

The plasma concentration of the [^{14}C]captopril rat plasma protein conjugate declined mono-exponentially, during the period of the experiment, with a half-life of 71 ± 2.2 min and an apparent volume of distribution of 14.1 ± 0.7 ml. The plasma concentrations of the captopril human plasma protein conjugate also declined mono-exponentially with a half-life of 137 ± 7.6 min, and an apparent volume of distribution of 16.5 ± 1.6 ml.

The tissue distribution of radioactivity, 3 hr after administration of [^{14}C]captopril, [^{14}C]captopril rat plasma protein conjugate and [^{14}C]captopril human plasma protein conjugate is shown in Fig. 4. There was a significantly greater accumulation of radioactivity in the liver, spleen and especially the lung

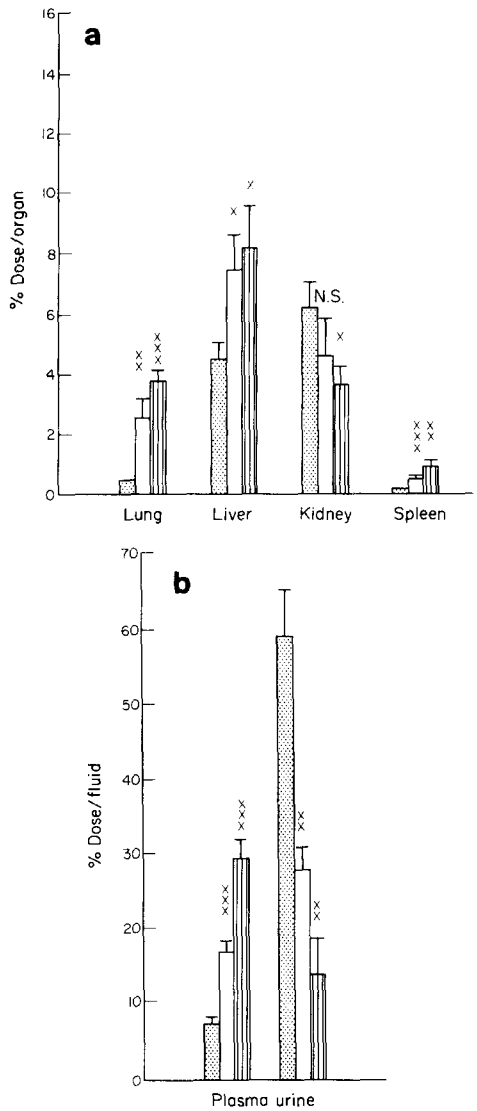

Fig. 4. Tissue distribution of ^{14}C radioactivity 3 hr after administration, to rats, of [^{14}C]captopril (■). [^{14}C]Captopril covalently bound to rat plasma proteins (□) and [^{14}C]captopril covalently bound to human plasma proteins (▥). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ using Student's *t*-test.

after administration of the [^{14}C]captopril protein conjugates than after infusion of free [^{14}C]captopril.

The amount of [^{14}C]captopril metabolites excreted in urine during the course of these experiments is presented in Table 1. After administration of free [^{14}C]captopril, [^{14}C]captopril itself was the major radioactive component in urine. However, after infusion of [^{14}C]captopril plasma protein conjugates [^{14}C]captopril cysteine mixed disulphide was the major radioactive component in urine.

DISCUSSION

The key functional group in captopril, both in terms of its metabolism and its covalent binding, is the free sulfhydryl group which can form covalent disulphide bonds with endogenous thiols *in vitro* [10]. It would appear from this, and other studies [10, 14, 15], that none of the other functional groups in captopril undergo biotransformation *in vivo*.

In previous studies it has been reported that captopril becomes extensively and covalently bound to human and rat plasma proteins when incubated *in vitro* [10]; covalent binding was determined by ultrafiltration and acid precipitation techniques. In the present work similar *in vitro* results were obtained using a novel equilibrium dialysis method [12] after extraction of metabolites with methanol. We found that [^{14}C]captopril also becomes covalently bound to plasma proteins *in vivo* in the rat, and 60 min after administration of [^{14}C]captopril approx. 34% of the dose was covalently bound to circulating plasma proteins (Fig. 2).

Therefore captopril has the potential to induce hypersensitivity reactions by acting as a hapten in immunogenic drug–protein conjugates [16, 18]. Captopril can also alter the biological properties of endogenous proteins by disrupting internal disulphide linkages [17]. It was therefore of interest to study the metabolic fate of [^{14}C]captopril covalently linked to plasma proteins.

The ease with which captopril forms covalent bonds with plasma proteins *in vitro* facilitated the preparation and purification of a [^{14}C]captopril rat plasma protein conjugate, in which 94% of the [^{14}C]captopril was covalently bound. After intravenous infusion, the drug–protein conjugate had a low apparent volume of distribution, indicating it was mainly distributed in the plasma compartment. During the time period studied, the plasma concentration of the drug–protein conjugate declined mono-exponentially, with a mean half-life of 71 min. Therefore, the plasma clearance of covalently bound [^{14}C]captopril was much greater than that of autologous or heterologous plasma proteins [19], sug-

Table 1. Metabolites of [^{14}C]captopril present in urine 3 hr after administration, to male Wistar rats, of [^{14}C]captopril rat plasma protein conjugate and [^{14}C]captopril human plasma protein conjugate

Material administered	% Dose in urine	% Urinary metabolites as CP	CPD	CP–Cys
[^{14}C]CP	59 ± 6	72 ± 4	12 ± 2	10 ± 1
[^{14}C]CP rat plasma protein conjugate	28 ± 3	18 ± 4†	6 ± 1*	67 ± 4†
[^{14}C]CP human plasma protein conjugate	14 ± 5	12 ± 2†	8 ± 2	57 ± 3†

$n = 4$, *$P < 0.05$, †$P < 0.001$.

gesting that dissociation of the drug–protein conjugate occurred *in vivo*. Examination of the tissue distribution of radioactivity, 3 hr after administration of the [^{14}C]captopril plasma protein conjugate, revealed the largest accumulation of radioactivity to be in urine, which contained a mean of 28% of the injected dose (Fig. 4). This value may slightly underestimate renal excretion of radioactivity because urine was obtained simply by terminal aspiration of the bladder. Chromatographic analysis showed that captopril cysteine mixed disulphide was the major (67%) radioactive component in urine but free [^{14}C]captopril and [^{14}C]captopril disulphide were also present in significant quantities.

In vitro studies have shown that captopril plasma protein disulphide linkages can be cleaved by thiols such as glutathione, cysteine and dithiothreitol [10]. Therefore, the *in vivo* dissociation of the drug–protein conjugate may occur in plasma where the glutathione concentration is 18 μM [20] and that of free cysteine 98 μM, and where the conjugate is largely confined. Displacement of captopril from plasma proteins leads to the formation of captopril glutathione mixed disulphide [15] which may either form free captopril with hepatic thioltransferases [15] or be hydrolysed by γ-glutamyltranspeptidase and dipeptidase, in the kidney, to give captopril cysteine mixed disulphide which was the major urinary metabolite of covalently bound captopril (Table 1).

A tentative scheme for the metabolism of captopril and the formation of captopril plasma protein conjugates, based on this and other work [10, 14, 15], is presented in Fig. 5. The major biotransformations of captopril involve mixed disulphide formation with endogenous thiols including plasma proteins, and methylation of the free sulfhydryl group. In the present context methylation may be regarded as a detoxication mechanism.

After administration of the [^{14}C]captopril plasma protein conjugate, there was a significantly greater accumulation of radioactivity in the liver, spleen and especially the lung than after corresponding experiments with [^{14}C]captopril. The altered tissue distribution may have immunological implications but it was not possible, in the present work, to determine whether this represented specific uptake of the captopril plasma protein conjugate by macrophages, which are present in high concentrations in these tissues.

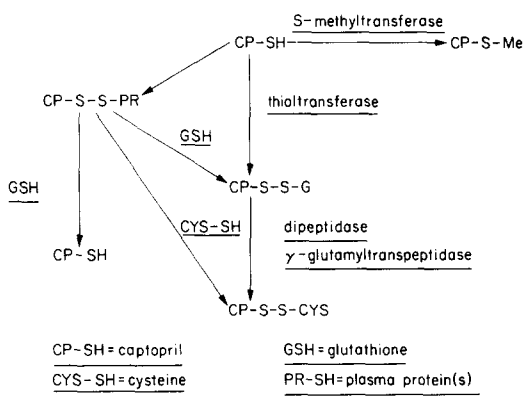

Fig. 5. Proposed metabolic scheme for captopril in the rat.

Finally, we investigated the metabolism of a [^{14}C]captopril human plasma protein conjugate in the rat. The distribution and metabolism of captopril administered covalently bound to human plasma proteins were similar to that of [^{14}C]captopril linked to rat plasma proteins. The major metabolite excreted in urine was [^{14}C]captopril cysteine mixed disulphide together with smaller amounts of free [^{14}C]captopril. [^{14}C]Captopril covalently bound to human plasma proteins had a similar volume of distribution to that of the rat plasma protein conjugate but was eliminated less rapidly from plasma. This probably reflects a difference in the rate of reaction with plasma gluthathione as there was also a reduction in urinary metabolites.

In conclusion, we have found that captopril forms covalent bonds with plasma proteins *in vitro* and *in vivo*. However, the covalent bonds formed, a disulphide linkage, is readily reversible *in vivo*; the relative rates of association and dissociation of captopril from plasma proteins may partly influence its ability to act as a hapten. Covalent binding to plasma proteins altered the tissue distribution of [^{14}C]captopril, but the immunological significance of this finding remains to be established.

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